

Group V allergens in grass pollens. II. Investigation of group V allergens in pollens from 10 grasses

F. MATTHIESEN and H. LØWENSTEIN

ALK Research, Hørsholm, Denmark

Summary

In an earlier study an allergen from *Phleum pratense* (timothy) pollen, *Phl p* V, has been isolated and physicochemically characterized. In this study *Phl p* V and immunochemically similar components from other grass pollens (group V allergens) have been investigated using immunoelectrophoretic techniques. To study the allergenic importance of the group V allergens, the allergenic compositions of 10 grass pollen extracts were investigated in crossed radioimmunoelectrophoresis (CRIE) using 20 sera from grass pollen-allergic donors. Group V allergens were identified using monospecific rabbit antibodies raised against *Phl p* V, anti-*Phl p* V, which react with other group V allergens usually producing dense precipitates in immunoelectrophoresis. In this way group V allergens were identified in eight extracts, and when present the precipitate corresponding to the group V allergen was the dominant IgE binding precipitate. All identified group V allergens bound IgE in at least 17 of the 20 investigated sera. Monospecific rabbit antibodies raised against the group I allergen of *Lolium perenne* (rye grass), anti-*Lol p* I, do not precipitate group V allergens, indicating that there are no immunochemical similarities between group I and group V allergens. In SDS-PAGE anti-*Phl p* V identifies IgE-binding components with molecular weights between 26 and 33 kD. In contrast, anti-*Lol p* I binds to components of slightly higher molecular weight. Apparently, the group V components are allergens that are physicochemically and immunochemically distinct from group I allergens.

Clinical and Experimental Allergy, Vol. 21, pp. 309–320. Submitted 8 August 1990; revised 28 November 1990; accepted 5 December 1990.

Introduction

A characterization of the group V allergen of *Phleum pratense* (timothy), *Phl p* V, has been reported in a previous paper [1]. Pollen allergens from different grass species are immunochemically similar when assayed in RAST inhibition experiments [2–5], by immunoelectrophoretic methods [6–7], and by techniques involving monoclonal antibodies [8–12]. Several of the grass pollen allergens have been purified. The group I allergen from *Lolium perenne* (rye grass) pollen, *Lol p* I, is one of the best studied grass pollen allergens. *Lol p* I consists of several immunochemically similar components with small charge heterogeneities and is of major allergenic importance [13–16]. Immunochemically and physicochemically similar group I allergens from other grass species have also been characterized [17–20] together with other allergens of less

importance, including *Lol p* II, *Lol p* III and *Lol p* IV [14–16, 21–23].

To determine the IgE-binding prevalence of group V allergens from different grasses, the allergenic composition of 10 grass pollen extracts has now been investigated using crossed radioimmunoelectrophoresis (CRIE). Immunochemical similarities between group V allergens from different grass species and between group V and I allergens from the same grass species have also been studied.

Materials and methods

Pollen extracts

Pollens from 10 grasses were obtained from Allergon (Uppsala, Sweden) as dried non-defatted material. The grass species investigated were the following (see Table 1 for abbreviations of extract names): *Alopecurus pratensis*, foxtail; *Avena elatior*, false oat; *Cynodon dactylon*, Ber-

Correspondence: Finn Matthiesen, ALK Research, Bøge Allé 10-12, DK-2970 Hørsholm, Denmark.

Table 1. Numbers of detected antigens in the 10 investigated grass pollen extracts as seen in CIE and CRIE

Pollen source	Extract abbreviation	Antibodies (no. of bleedings before use)	Antigens CIE*	Allergens CRIE†
<i>Alopecurus pratensis</i>	<i>Alop</i>	10	25	8
<i>Avena elatior</i>	<i>Avee</i>	10	22	6
<i>Cynodon dactylon</i>	<i>Cynd</i>	33	34	8
<i>Dactylis glomerata</i>	<i>Dacg</i>	10	27	8
<i>Festuca pratensis</i>	<i>Fesp</i>	10	21	6
<i>Lolium perenne</i>	<i>Lolp</i>	20	31	9
<i>Phleum pratense</i>	<i>Phlp</i>	124	30	11
<i>Phragmites communis</i>	<i>Phrc</i>	10	31	3
<i>Poa pratensis</i>	<i>Poap</i>	10	25	9
<i>Secale cereale</i>	<i>Secc</i>	10	22	7

* Number of precipitates in CIE.

† Number of IgE binding precipitates in CRIE.

muda grass; *Dactylis glomerata*, orchard grass; *Festuca pratensis*, meadow fescue; *Lolium perenne*, rye grass; *Phleum pratense*, timothy grass; *Phragmites communis*, reed; *Poa pratensis*, Kentucky blue grass; *Secale cereale*, cultivated rye. According to the manufacturer's certificates, each pollen sample contained approximately 89–96% pure grass pollen grains of the specified grass species and approximately 5–10% other plant parts from the flowers of the same grass. Detectable foreign pollens constituted less than 0.5% (usually less than 0.1%).

The pollens were stored and extracted as described elsewhere [1] to obtain freeze-dried, allergen-containing extracts. Analysis for protein content, as described by Lowry *et al.* [24], using bovine serum albumin as a reference revealed 50–80% (w/w) of the grass pollen extracts to be of protein nature.

Polyspecific rabbit antisera

Specific anti-grass pollen antibodies were raised by immunizing three rabbits with one of each specific grass pollen extract (approximately 5 µg/injection in Freund's incomplete adjuvant). The monthly immunizations, the monthly bleedings and subsequent isolation of the immunoglobulins by ammonium sulphate fractionation were performed as described [1].

Immunoglobulins against seven of the grass pollen extracts were isolated after 10 bleedings. Immunizations with three grasses were started earlier than the rest. Therefore, immunoglobulins against *Cynd*, *Lolp* and *Phlp* were isolated after more bleedings. The antibodies, concentrated 5–10 times relative to their concentrations in serum, were stored as described [1].

Monospecific rabbit antisera

Monospecific antisera against group V and group I allergens were prepared by immunizing rabbits with *Phl p* V and *Lol p* I. *Phl p* V was purified in our laboratory as described [1], and *Lol p* I was kindly supplied by Dr D. G. Marsh. The amount of allergen in each monthly injection was estimated to approximately 1 µg. Antibodies against *Phl p* V (anti-*Phl p* V) and *Lol p* I (anti-*Lol p* I) were isolated after five and six monthly bleedings, respectively. The antibodies were treated and stored as described for the polyspecific antibodies.

Sera

Sera from 20 donors resident in Denmark who had allergic rhinitis during the grass pollen season were investigated. Preliminary, all sera were tested by RAST according to Ceska *et al.* [25]. All patients revealed a strong positive response (> RAST class 4) in all performed grass RAST experiments. None of the patients had previously undergone specific immunotherapy. A serum pool of 1000 Danish individuals was used as a control serum.

Immunoelectrophoresis

Analyses using crossed immunoelectrophoresis (CIE) and fused rocket immunoelectrophoresis (FRIE) were performed at pH 8.6 using 1% agarose gels (type HLA; Litex, Copenhagen, Denmark) as described elsewhere [1]. Details of the electrophoresis are given in Results.

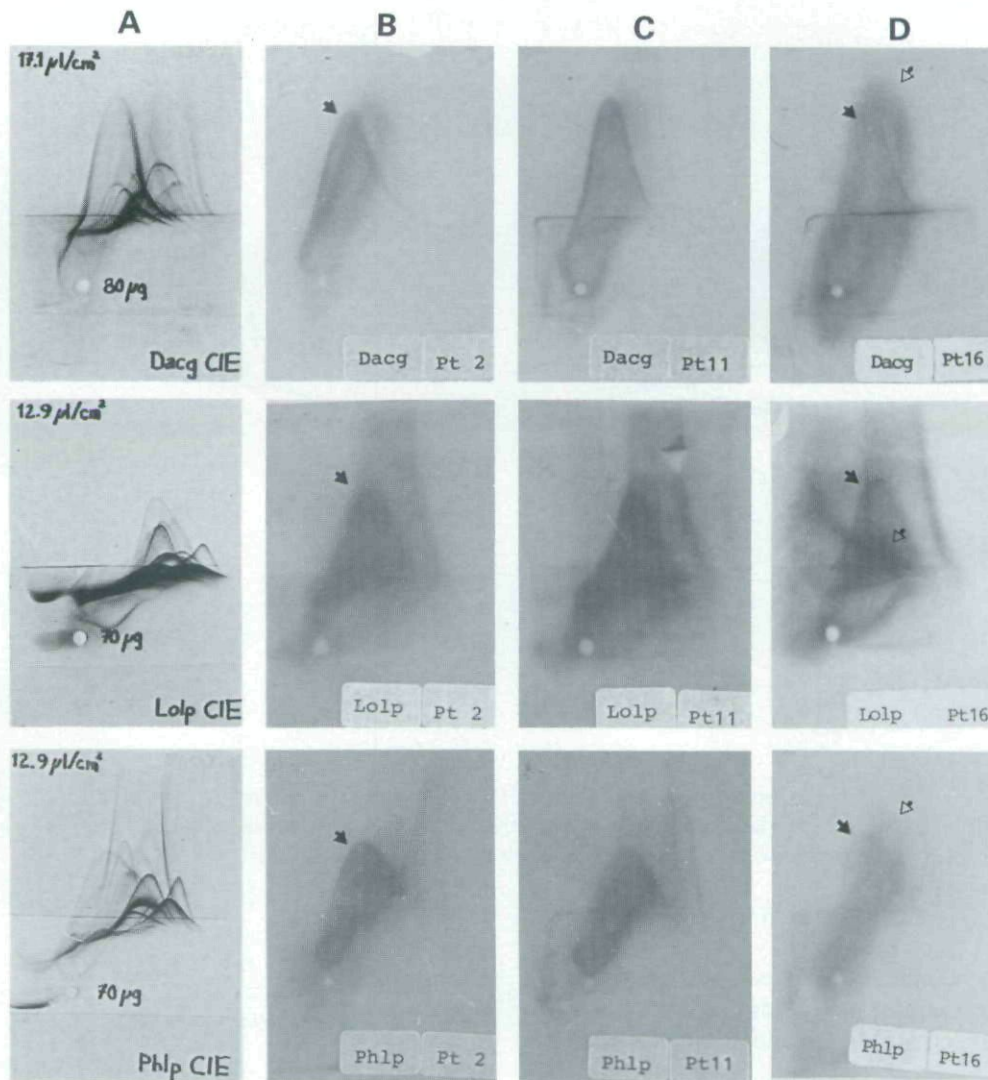


Fig. 1. CRIE studies: allergen detection in three selected grass pollen extracts *Dacg*, *Lolp* and *Phlp*. (A) CIE plates; (B–D) selected CRIE autoradioprints with sera Pt. 2 (mainly responding to group V allergens), Pt. 11 and Pt. 16. CIE/CRIE on 5×7 -cm plates: first-dimension electrophoresis for 30 min at 10 V/cm, second-dimension electrophoresis overnight at 2 V/cm. Amounts of extract and polyspecific antibodies as shown on figures. Group I and group V allergens are indicated with blank and solid arrows.

Ten crossed radioimmuno-electrophoretic studies (CRIE studies) were performed, each with 20 sera from patients allergic to temperate grasses, a pool of the sera, and a negative control serum (a 1000-donor pool). The CRIE plates were prepared as described elsewhere [1]. The dried plates were autoradiographed at -20°C for 10 days. The radiostaining of the X-ray films (Kodak X-omat L) was quantified as follows: the degree of radiostaining of individual precipitates in the plates was evaluated by visual comparison of CRIE autoradiographies with autoradiographies of ^{125}I -anti-IgE single radial radioimmunodiffusion (SRRID) rings. The control SRRID plates were prepared as follows: 50- μl aliquots of

two-fold dilutions (nine in all) of ^{125}I -anti-IgE were mixed with 5 μl of rabbit IgG (Dakopatts, Glostrup, Denmark). The SRRID were performed with 5 μl of each dilution (7–0.03 nCi) for 48 hr on 5×5 -cm² glass plates coated with agarose containing 3 $\mu\text{l}/\text{cm}^2$ of swine anti-rabbit IgG (Dakopatts). In each X-ray film cassette one SRRID plate was autoradiographed together with 15 or less CRIE plates. Each CRIE study was performed separately. The radiostained precipitates were assigned a score N, according to $N = 32/D$, where D is the dilution of ^{125}I -anti-IgE in the SRRID ring with corresponding radiostaining intensity (e.g. a dilution of 1 + 15 gives $N = 2$). The degree of radiostaining of the precipitates in each CRIE study was

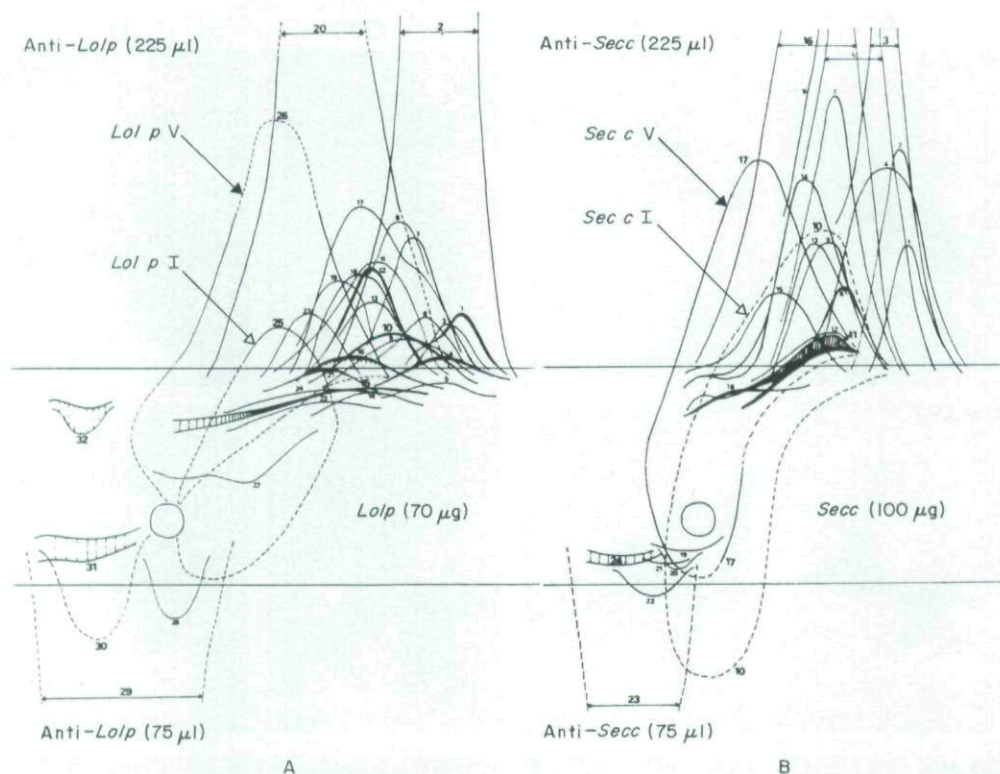


Fig. 2. CIE/CRIE reference drawings: interpretations of the CIE/CRIE precipitation patterns are shown for (A) *Lolp*; (B) *Secc*. The precipitates are numbered from right to left according to peak position. Precipitates which are only visible in CRIE are dotted. Group I and group V allergens are indicated with solid and blank arrows.

evaluated independently by two persons who visually compared the intensity of the radiostained precipitation arcs with the intensity of the corresponding SRRID rings.

SDS-PAGE, electroblotting and immunoblotting

SDS-PAGE, electroblotting and IgE immunoblotting were performed as described elsewhere [1].

Electroblots for IgG immunoblot were incubated overnight with 50 µl/sample rabbit antiserum diluted 1/1000 (v/v) in incubation buffer (0.05% Tween 20 in 0.05 M Tris and 0.15 M NaCl, adjusted to pH 10.3), followed by three washings in incubation buffer, followed by 3 hr of incubation in alkaline-phosphatase-conjugated swine anti-rabbit IgG antibodies (Dakopatts) diluted 1/1000 (v/v) in incubation buffer. The blots were developed by using freshly prepared alkaline phosphatase substrate (0.11 mM nitroblue tetrazolium, 0.14 mM 5-bromo-4-chloro-3-indolylphosphate, 3.6 mM MgCl₂ in 0.1 M ethanolamin buffer, adjusted to pH 9.6). Molecular weight markers were the same as used in the accompanying study [1].

Results

Allergenic composition of extracts

The antigenic and allergenic compositions of 10 grass pollen extracts were studied by CRIE using polyspecific rabbit antibodies and 20 sera from Danish grass-pollen-allergic donors.

The numbers of antigens and allergens detected in the extracts are reported in Table 1, and representative CRIE patterns (with corresponding CIE precipitation patterns) are shown in Fig. 1. The number of detected antigens vary from 21 in *Fesp* to 34 in *Cynd*. A characteristic precipitation pattern is observed in all extracts; e.g. usually a dense arc is precipitated just to the right of the application slit in the anodic gel, most antigens precipitate solely in the anodic gel, and only few antigens (from one to five antigens) precipitate solely in the cathodic gel.

Using sera from the 20 grass-pollen-sensitized subjects, IgE binding to approximately every third or fourth of the detectable antigens is observed in most of the extracts. The number of allergens detectable in CRIE varies from three in *Phrc* to 11 in *Phlp*. Allergens are precipitated in both the anodic and cathodic gels. The most intensely

Table 2. Evaluation of the CRIE experiment of *Secale cereale*. CRIE scores of individual sera to antigens in the pollen extract *Secc* (Ag10 and Ag17 represent *Sec c I* and *Sec c V*)

Serum no.	Ag 3	Ag 10	Ag 11	Ag 16	Ag 17	Ag 21	Ag 22
Pt. 1	—	1	5	2	3	—	—
Pt. 2	—	—	5	—	4	1	2
Pt. 3	—	2	5	—	5	2	—
Pt. 4	—	1	5	—	4	—	2
Pt. 5	—	1	5	—	5	—	—
Pt. 6	—	3	5	1	4	1	—
Pt. 7	—	2	2	—	1	—	—
Pt. 8	—	3	5	1	6	—	2
Pt. 9	—	2	4	—	4	—	—
Pt. 10	2	1	4	—	1	—	1
Pt. 11	2	2	5	—	5	—	—
Pt. 12	—	—	2	—	—	—	1
Pt. 13	—	2	2	—	1	1	—
Pt. 14	—	2	4	—	4	—	—
Pt. 15	—	2	4	2	3	1	1
Pt. 16	—	4	5	—	5	1	2
Pt. 17	—	—	4	—	5	—	—
Pt. 18	—	2	2	2	4	—	—
Pt. 19	—	1	2	—	4	—	—
Pt. 20	—	2	5	—	5	2	—
Pool*	—	2	5	—	4	—	—
Non-specific binding†	—	—	2	—	—	—	—

* Pool of the 20 individual sera

† Non-specific binding as revealed in the 1000-donor pool (negative control).

radiostained allergen always precipitates around the application slit, however, with the main part in the anodic gel.

In each pollen extract the individual blue-stained or IgE-binding precipitates were numbered from right to left according to peak position (as shown for *Lol p* and *Secc* in Fig. 2), and the IgE binding of each precipitate was evaluated as described in Materials and Methods. The result of such an evaluation is shown in Table 2.

Using a 1000-donor pool of Danish donors, non-specific IgE binding is observed to a single precipitate in six extracts (e.g. Ag11 in *Secc*, Fig. 2B). This precipitate is dense and heavily blue-stained in CIE. In all CRIE performed with individual sera, the precipitate is radiostained indicating the binding of anti-IgE.

In Table 3 the individual IgE binding precipitates of all grass pollen extracts are listed together with their migration in CIE/CRIE and the number of sera containing IgE with specificity against the allergen.

Identification of allergens

The presence of group V and group I allergens in the 10 extracts was investigated using monospecific antibodies raised against *Phl p V* and *Lol p I* by CIE/CRIE and SDS-PAGE immunoblot.

The components were identified in the CIE/CRIE precipitation pattern by performing CIE with the respective monospecific antibodies in the intermediate gel (Fig. 3 and Table 3). Anti-*Phl p V* precipitates an IgE-binding component in all extracts except *Cynd* and *Phrc*, and anti-*Lol p I* precipitates an IgE-binding component in all extracts except *Cynd*. When present, the anti-*Phl p V* precipitate is the most dominantly radiostained precipitate in CRIE. Both anti-*Phl p V* and anti-*Lol p I* bind allergens that migrate into the anodic gel; however, usually the corresponding precipitates are continuing around the application slit (Fig. 1). In three extracts (*Avee*, *Dacg* and *Poap*), the anti-*Phl p V* precipitate

Table 3. Results of the 10 performed CRIE studies. Relative migration, IgE prevalences and proposed immunochemical similarities (as identified using anti-*Phl p V* and anti-*Lol p I*) are stated.

Antigen no.*	Relative migration†	IgE binding prevalence‡			Non-specific binding§	Comments	Proposed name¶	Mol. wt (kD) SDS-PAGE**
		(a)	(b)	(c)				
<i>Alopecurus pratensis</i> (foxtail)								
Ag1	4.3	12	12	0	—			
Ag9	3.1	3	3	0	—			
Ag10	2.9	19	18	1	—			
Ag13	2.8	20	1	19	+	Aggregated antigens?		
Ag16	2.4	3	3	0	—			
Ag17	2.4	6	6	0	—			
Ag21	1.0	17	16	1	—	Group V component	<i>Alo p V</i>	
Ag23	−0.5	18	15	3	—	Group I component?	<i>Alo p I</i>	32
<i>Avena elatior</i> (false oat)								
Ag8	2.1	12	12	0	—			
Ag14	1.6	20	13	7	—	Group I component	<i>Ave e I</i>	35
Ag17	1.4	20	13	7	+	Aggregated antigens?		
Ag19	1.0	19	11	8	—	Group V component	<i>Ave e V</i>	26/28
Ag21	−0.4	10	10	0	—			
Ag22	−0.4	12	12	0	—			
<i>Cynodon dactylon</i> (Bermuda grass)								
Ag3	4.1	11	10	1	—			
Ag5	3.8	1	1	0	—			
Ag8	3.5	1	1	0	—			
Ag15	2.8	1	1	0	—			
Ag18	2.8	2	2	0	—			
Ag24	1.9	13	8	5	—	Aggregated antigens?		
Ag25	1.3	6	6	0	—			
Ag26	1.0	20	6	14	—	Group I component	<i>Cyn d I</i>	32
<i>Dactylis glomerata</i> (orchard grass)								
Ag13	1.6	1	1	0	—			
Ag14	1.5	20	11	9	—	Group I component	<i>Dac g I</i>	34
Ag17	1.5	16	16	0	—	Aggregated antigens?		
Ag18	1.5	6	6	0	—			
Ag23	1.0	20	10	10	—	Group V component	<i>Dac g V</i>	26/28
Ag25	−0.2	7	7	0	—			
Ag26	−0.4	6	6	0	—			
Ag27	−1.0	11	11	0	—			
<i>Festuca pratensis</i> (meadow fescue)								
Ag4	1.3	2	2	0	—			
Ag9	1.1	12	12	0	—	Aggregated antigens?		
Ag10	1.0	18	18	0	—			
Ag11	1.0	19	19	0	—	Group V component	<i>Fes p V</i>	
Ag18	0.8	18	18	0	—	Group I component?	<i>Fes p I</i>	34
Ag21	−0.4	12	12	0	—			
<i>Lolium perenne</i> (rye grass)								
Ag2	2.5	2	2	0	—			
Ag9	2.1	1	1	0	—			
Ag15	1.7	20	18	2	+	Aggregated antigens?		

Lolium perenne (rye grass)—Continued

Ag20	1.4	16	8	8	—			
Ag25	1.0	18	10	8	—	Group I component	<i>Lol p I</i>	33
Ag26	1.0	17	2	15	—	Group V component	<i>Lol p V</i>	25/27
Ag29	−0.4	2	2	0	—			
Ag30	−0.8	13	10	3	—			
Ag31	−0.8	4	4	0	—			

Phleum pratense (timothy)

Ag4	2.6	6	6	0	—			
Ag5	2.5	14	14	0	—			
Ag11	2.0	18	18	0	—			
Ag13	1.8	20	15	5	—	Aggregated antigens?		
Ag14	1.7	15	15	0	—			
Ag16	1.6	2	2	0	—			
Ag17	1.6	12	12	0	—	Group I component	<i>Phl p I</i>	34
Ag25	1.0	19	13	6	—	Group V component	<i>Phl p V</i>	29/33
Ag26	ND	2	2	0	—			
Ag27	−0.7	12	11	1	—			
Ag28	−1.2	15	15	0	—			

Phragmites communis (reed)

Ag4	4.6	5	5	0	—			
Ag22	2.8	20	20	0	+	Aggregated antigens?		
Ag29	1.0	20	20	0	—	Group I component	<i>Phr c I</i>	

Poa pratensis (blue grass)

Ag2	3.0	6	5	1	—			
Ag14	2.0	16	10	6	—			
Ag18	1.5	20	1	19	+	Aggregated antigens?		
Ag19	1.2	19	11	8	—	Group I component	<i>Poa p I</i>	33
Ag20	1.0	20	5	15	—	Group V component	<i>Poa p V</i>	26/28
Ag21	−0.2	8	7	1	—			
Ag22	−0.5	20	10	10	—			
Ag23	−0.9	20	16	4	—			
Ag24	−1.0	6	6	0	—			

Secale cereale (cultivated rye)

Ag3	3.0	2	2	0	—			
Ag10	1.9	17	17	0	—	Group I component	<i>Sec c I</i>	30/33
Ag11	1.9	20	5	15	+	Aggregated antigens?		
Ag16	1.8	5	5	0	—			
Ag17	1.0	19	12	7	—	Group V component	<i>Sec c V</i>	
Ag21	−1.1	7	7	0	—			
Ag22	−1.3	7	7	0	—			

* According to the CIE/CRIE reference drawings.

† In CIE/CRIE compared to the group V allergen (migration = 1). In *Cynd* and *Phrc* relative to the group I allergen. Negative values indicate cathodic migration.

‡ IgE binding prevalences in CRIE: (a) Total number of sera revealing IgE that binds to the precipitate; (b) number of sera with low scores 1–4; and (c) number of sera with high scores 5–8.

§ Presence of non-specific binding to the antigen in CIE/CRIE.

¶ Proposed names (according to the IUIS nomenclature) [34].

** Molecular weight as determined in SDS-PAGE IgG immunoblots; the molecular weight of the band(s) identified with anti-*Phl p V* and anti-*Lol p I* is shown.

ND, not determined.

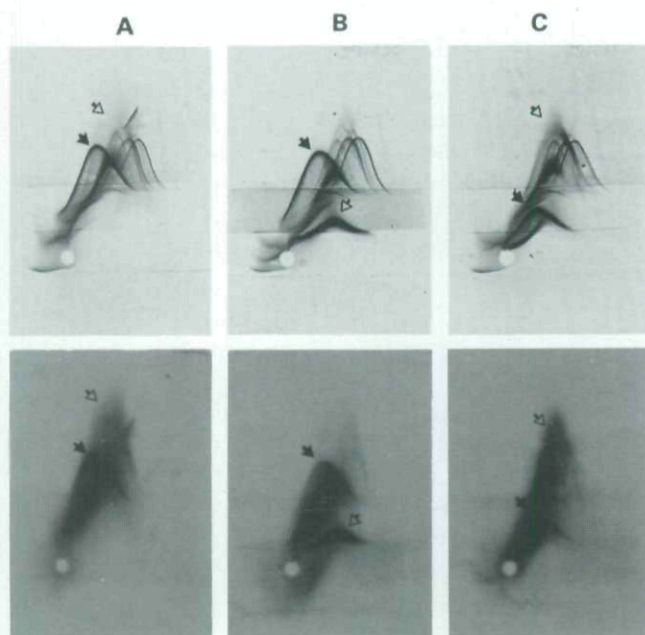


Fig. 3. Identification of group I and group V components in CIE/CRIE: identifications in CIE (top) and CRIE (bottom) of *Ave e I/Ave e V* from *Avee*. CIE/CRIE as in Fig. 1, except that a pool of the 20 investigated sera was used in CRIE. (A) Intermediate gel empty; (B) intermediate gel contains 20 $\mu\text{l}/\text{cm}^2$ anti-*Lol p I*; (C) intermediate gel contains 5 $\mu\text{l}/\text{cm}^2$ anti-*Phl p V*. *Ave e I* and *Ave e V* are indicated with blank and solid arrows.

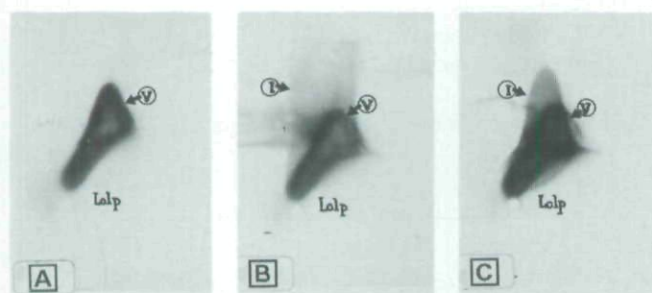


Fig. 4. Investigation of immunochemical differences between group I and group V allergens: *Lol p* as seen in CRIE using solely monospecific antibodies as precipitating reagents. Upper and lower gel contain 5.7 $\mu\text{l}/\text{cm}^2$ anti-*Phl p V* mixed with: (A) no anti-*Lol p I*; (B) 2.6 $\mu\text{l}/\text{cm}^2$ anti-*Lol p I*; (C) 5.7 $\mu\text{l}/\text{cm}^2$ anti-*Lol p I*. Two distinct allergens are precipitated using anti-*Phl p V* and anti-*Lol p I*.

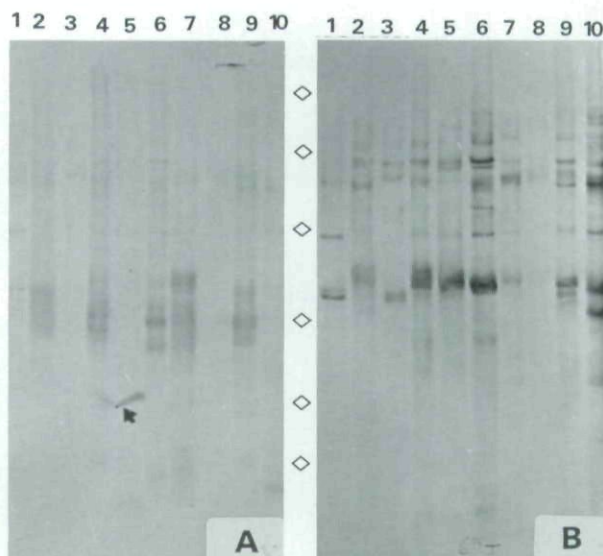


Fig. 5. Identification of group I and group V allergens in SDS-PAGE IgG immunoblot: IgG immunoblots using: (A) anti-*Phl p V*; (B) anti-*Lol p I*. Grass pollen extracts applied (5 μg) are 1, *Alo p*; 2, *Ave e*; 3, *Cyn d*; 4, *Dacg*; 5, *Fes p*; 6, *Lol p*; 7, *Phl p*; 8, *Phrc*; 9, *Poa p*; 10, *Secc*. The arrow indicates an artefact.

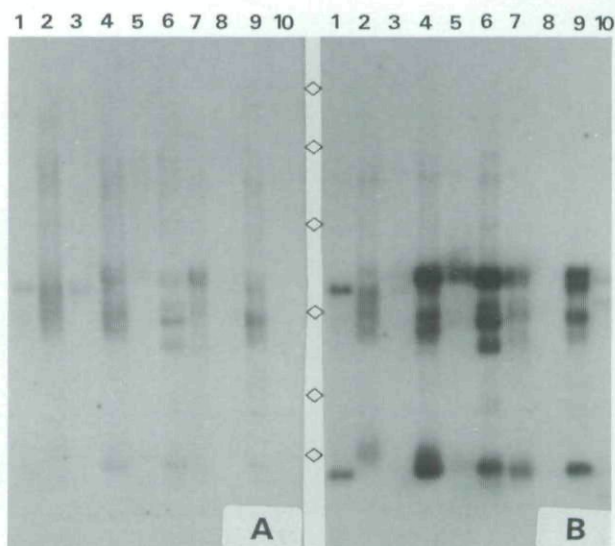


Fig. 6. Group I and group V allergens in SDS-PAGE IgE immunoblot: IgE immunoblots using serum Pt. 2 (left) which mainly identifies group V allergens, and serum Pt. 16 (right) which identifies both group I and group V allergens. Pollen extracts as in Fig. 5.

consists of two precipitation arcs (Fig. 3). When mixtures of anti-*Phl p* V and anti-*Lol p* I are used in CIE, two immunochemically distinct components are precipitated (Fig. 4).

In SDS-PAGE immunoblot the monospecific antibodies bind to many bands. The dominant bands, however, also bind IgE in sera from grass pollen sensitized donors in the patient panel (Figs 5 and 6). Anti-*Phl p* V clearly binds to components in *Avee*, *Dacg*, *Lolp*, *Phlp* and *Poa p*, and in all extracts the antibodies bind to more than one band (Table 3). The components thus defined have molecular weights of approximately 26–33 kD. Anti-*Lol p* I binds to components in all extracts with the possible exception of *Phrc*, and defines components with molecular weights of approximately 30–35 kD. In *Secc*, anti-*Lol p* I clearly binds to two distinct bands in this range.

Discussion

Allergenic composition of grass pollen extracts

In this study the antigenic and allergenic compositions of 10 grass pollen extracts have been investigated by the immunoelectrophoretic techniques CIE and CRIE using polyspecific rabbit antibodies and sera from 20 grass-pollen-sensitized human individuals.

All grass species belong to the grass family *Poaceae* (*Gramineae*). Eight of the investigated grasses are taxonomically closely related in the grass subfamily *Pooideae* [26] and commonly used in Europe for *in vitro* diagnostics. *Phragmites communis* (reed) and *Cynodon dactylon* (Bermuda grass) belong to the subfamilies *Arundinoideae* and *Chloridoideae*, and they were included in the study because of their taxonomical diversity from most well-studied grasses.

The CIE and CRIE techniques are extremely applicable to detect individual patient responses: the proteins are treated gently, immunochemically distinct components are easily distinguished and immunochemical cross-reactivity is easily detected. However, detection and identification of allergens are dependent on the rabbit antibodies, and mixtures of a high number of bleedings from several rabbits must be used to ensure a high quality of antibodies. Therefore, in this study three rabbits were immunized with each extract to average the individual rabbit responses, and the antibodies were used only after at least 1 year of immunization (more than 10 bleedings).

CIE/CRIE patterns of grass pollen extracts generally look much the same from investigation to investigation. In this study the observed CIE/CRIE precipitation patterns reveal many similarities to precipitation patterns from other studies of grass pollens [6,7,27–29]. The precipitates of all 10 grass pollen extracts reveal many

common features, indicating that the precipitated antigens have similar physicochemical properties, e.g. allergens with high IgE-binding prevalence are generally situated around the application slit with the major part in the anodic gel.

All the 20 investigated individual serum samples contained IgE against at least one (usually more than one) component in all 10 investigated grass pollen extracts. All sera contained IgE which bound specifically to components in the taxonomically distinct grasses, *Phragmites communis* and *Cynodon dactylon*. The presence of IgE towards components in all the investigated grass pollens cannot be due to a broad sensitization with all pollens. All the temperate grasses investigated are present in the Danish environment (with *Phleum pratense* and *Dactylis glomerata* as the dominating species), and the Danish grass-pollen-allergic donors are potentially sensitized with pollen allergens from all these species. However, the Danish donors also revealed IgE against the subtropic *Cynodon dactylon* which is neither cultured, nor naturally occurring in Denmark. The observed crossreactivity is presumably due to the existence of common IgE epitopes on antigens of even taxonomically rather distinct grasses. The existence of such common epitopes between *Cynodon dactylon* and temperate grasses as *Koeleria* and *Elymus* spp. has been proposed in a recent study by Schumacher *et al.* [30]. The observation verifies that grass pollen extracts are composed of immunochemically related components which are similar with respect to the IgE immune system in humans.

In all extracts, a heavily blue-stained precipitate is present just above the application slit. In six of the extracts this component binds IgE from all investigated sera, but at the same time it reveals non-specific radio-staining after incubation with the negative control serum. Therefore, it is suggested that these precipitates are capable of binding IgE non-specifically. It is assumed that this sort of precipitate consists of aggregates of different antigens (including allergens) which have not been properly dissolved during the solution procedure, possibly because of strong non-specific ionic interactions. The assumption is supported by the observations that the precipitates are of high molecular weight (> 70 kD, as seen in gel filtration experiments) [1,29], that the precipitate is slightly affected by the monospecific antibodies, and that no corresponding SDS-PAGE band has been found, presumably because the treatment destroys the aggregates.

Identification of allergens

Several components have been reported as important allergens in grass pollen extracts. Group I components, molecular weights in SDS-PAGE 27–36 kD [14–18,31],

the group II and III components, both 11 kD in SDS-PAGE [14,16,23], and group IV components, 50–60 kD in SDS-PAGE [21,22] have been characterized in grass pollens, preferably from *Lolium perenne*.

The most dominant IgE-binding component in the *Phleum pratense* CRIE, *Phl p V*, has recently been described and monospecific rabbit antibodies raised against it [1]. When anti-*Phl p V* is placed in the intermediate gel in CIE, the most dominant IgE binding precipitate in eight of the grass pollen extracts (*Alop*, *Avee*, *Dacg*, *Fesp*, *Lolp*, *Phlp*, *Poap* and *Secc*) are precipitated in the intermediate gel. Earlier preliminary studies indicated that this IgE binding component was not a group I allergen (which could be assumed from molecular weight studies). To confirm this, antibodies against *Lol p I* (kindly supplied by Dr D. G. Marsh) were prepared, and simultaneous tests with anti-*Phl p V* and anti-*Lol p I* were performed. These studies confirm that *Phl p V* is not a group I allergen: different allergenic components are precipitated in all grasses when CIE is performed with monospecific antibodies in the intermediate gels (Fig. 3), and mixtures of the monospecific antibodies precipitate two immunochemically distinct IgE-binding components in *Lolium perenne* (Fig. 4).

In *Phrc* and *Cynd* it is not possible to precipitate an allergen with anti-*Phl p V*. This might be due to the lack of immunochemical similarities between *Phl p V* and the proposed group V allergens in these taxonomically rather distinct grasses, or it might be due to a stoichiometric low amount (or simply absence) of the allergens in the extracts.

In some extracts the anti-*Phl p V* precipitates are double, indicating that more than one antigen is drawn into the intermediate gel. These double precipitates are present when the group V precipitate is very dense in the CIE pattern (*Avee*, *Dacg* and *Poap*), and might be due to the ability of anti-*Phl p V* antibodies to distinguish between isomers of group V allergens from these grasses. When two arcs are present, they are partly coinciding indicating that the precipitated antigens are immunochemically similar.

In CIE, the anti-*Lol p I* antibodies precipitate an IgE-binding component in all extracts except *Cynd* and *Phrc*. In *Avee*, *Dacg*, *Lolp* and *Poap* the group I precipitates are well defined, but in *Alop*, *Fesp*, *Phlp* and *Secc* the group I precipitates are only putatively identified (Fig. 1 and Table 3), because the respective polyspecific antibodies only precipitate the group I allergens very weakly. Generally, the group I precipitates are diffuse and partly hidden by the well defined group V precipitates. However, the group I components usually migrate further towards the anode in the first-dimension electrophoresis and are thereby slightly separated from the group V components.

Immunoelectrophoretic techniques as CIE seem not to be very useful for detection of group I allergens. Except for *Cyn d I*, all group I allergens are poorly precipitated with the polyspecific antibodies raised against whole pollen extracts. Although rabbit anti-*Lol p I* was capable of making well defined precipitates with group I allergens, generally rabbits seem to respond rather poorly to group I allergens. In contrast, rabbits seem to respond strongly to group V allergens and immunoelectrophoretic techniques seem to be excellent for detection of group V allergens.

Because of the different titre of the applied rabbit antibodies to group I and group V allergens, it is impossible to elucidate the relative allergenic importance of these allergens from the immunoelectrophoretic observations. Consequently, such determinations can not be performed by using techniques relying on rabbit antibodies.

To confirm the CIE/CRIE results, SDS-PAGE immunoblots with the monospecific antibodies and selected sera from the patient panel were made. The technique of SDS-PAGE is not dependent on antibodies. However, the proteins are heat treated, denatured and reduced, and even though a certain degree of renaturation occurs after electroblotting, the proteins are often not presented in their native forms. If the IgG or IgE epitopes of the allergenic component are disturbed by SDS-PAGE, the component will not be detected in the immunoblots.

Anti-*Phl p V* and anti-*Lol p I* bind to different components in all extracts, even though much nonspecific binding is also present (Fig. 5). However, the dominant bands identified by the antibodies also bind IgE as seen in immunoblots using sera from the patient panel (Fig. 6). The anti-*Phl p V* antibodies bind several components in the molecular weight range of 26–33 kD, while anti-*Lol p I* binds mainly to components of slightly greater size of 30–35 kD. Anti-*Lol p I* also bind to some high molecular weight bands not identified by anti-*Phl p V*; presumably, this binding is due to impurities in the allergen preparations used for immunization.

The observation that the antibodies identify different allergenic components is emphasized by the correlation between CRIE and IgE immunoblots: sera which contain IgE mainly against the group V precipitate in CRIE (e.g. Pt. 2) also reveal IgE that binds mainly to the group V bands in immunoblots; in contrast, sera containing IgE against both group I and V allergens (e.g. Pt. 16) identify both the group I and V bands in SDS-PAGE immunoblot (Fig. 6).

In *Alop*, *Fesp* and *Secc* group V components are identified in CIE/CRIE, but not in the immunoblot studies. It is assumed that the group V allergens during the SDS-PAGE treatment have been modified or degraded so that the IgG and IgE epitopes have disap-

peared. Similar loss of epitopes has been reported earlier in studies of tree pollen extracts [32].

In *Phrc* and *Cynd* the major allergenic components, *Phr c I* and *Cyn d I*, are physicochemically similar to the group I allergens [29,33]. However, using polyclonal antibodies in CIE/CRIE, *Phr c I* and *Cyn d I* are only weakly precipitated with anti-*Lol p I*, indicating a considerable immunochemical dissimilarity. In SDS-PAGE IgG immunoblot, anti-*Lol p I* clearly identifies *Cyn d I*, indicating that some immunochemical resemblance between *Cyn d I* and *Lol p I* is in fact present. In similar studies using monoclonal antibodies, this has also been observed [9,31], and immunochemical similarities are indeed indicated by the IgE response of Danish grass-pollen-allergic individuals.

Group V allergens

In CIE/CRIE the group V allergens are identified in all the eight grasses from the grass subfamily *Pooideae*, the allergens are for the present named *Alo p V*, *Ave e V*, *Dac g V*, *Fes p V*, *Lol p V*, *Phl p V*, *Poa p V* and *Sec c V* (Table 3). The anti-*Phl p V* antibodies crossreact strongly with these group V components, indicating a high degree of epitope similarity. This resemblance also indicates that parts of the molecular structure might have been preserved during the evolution and that these parts (and possibly the whole molecule) might have an important physicochemical or biological function.

The precipitates of *Fes p V* and *Lol p V* are weakly blue-stained (Fig. 1) and only detectable after radiostaining in CRIE. Other group V allergens, like *Dac g V* and *Poa p V*, are identified in extremely dense precipitates. Most probably, the variable detection might be due to a simple stoichiometric variation in the amounts of group V components in the investigated grass pollen extracts.

In the CRIE studies all the identified group V components bind IgE from at least 17 of the 20 of the investigated sera, and they are all easily detected in CRIE. The immunochemical divergence of group V and group I allergens has been hidden by the fact that the heavily radiostained group V precipitates usually are overlaying the rather badly defined group I precipitates. However, after reevaluation of elder CRIE investigations from our laboratory, the group I component is recognized as the group V 'shadow' in the precipitation patterns, for example Ag13 in an early investigation on *Secale cereale* [27].

From these results we conclude that the anti-*Phl p V* antibodies identify an immunochemically distinct group of grass pollen allergens different from earlier described grass pollen allergens.

Acknowledgments

We wish to thank Ms Lotte Friberg, Ms Lone Fink and Ms Kirsten Bache Billesbølle for their skilful technical assistance.

References

- 1 Matthiesen F, Löwenstein H. Group V allergens in grass pollens. I. Purification and characterization of the group V allergen from *Phleum pratense* pollen, *Phl p V*. Clin Exp Allergy 1991; 21:297-307.
- 2 Marsh DG, Haddad ZH, Campbell DH. A new method for determining the distribution of allergenic fractions in biological materials: its application to grass-pollen extracts. J Allergy 1970; 46:107-21.
- 3 Bernstein IL, Perera M, Gallagher J, Michael JG, Johansson SGO. In vitro cross-allergenicity of major aeroallergenic pollens by the radioallergosorbent technique. J Allergy Clin Immunol 1976; 57:141-52.
- 4 Leiferman KM, Gleich GJ. The cross-reactivity of IgE antibodies with pollen allergens. I. Analysis of various species of grass pollens. J Allergy Clin Immunol 1976; 58:129-39.
- 5 Martin BG, Mansfield LE, Nelson HS. Cross-allergenicity among the grasses. Ann Allergy 1985; 54:99-104.
- 6 Löwenstein H. Immunological partial identity and in vitro inhibitory effect of two major pollen allergens to whole pollen extract of four grasses. Int Arch Allergy Appl Immunol 1978; 57:379-83.
- 7 Chakrabarty S, Löwenstein H, Ekramoddoullah AKM, Kisil FT, Schon AH. Detection of cross-reactive allergens in Kentucky bluegrass pollen and six other grasses by crossed radioimmuno-electrophoresis. Int Archs Allergy Appl Immunol 1981; 66:142-57.
- 8 Singh MB, Knox RB. Grass pollen allergens. Antigenic relationships detected using monoclonal antibodies and dot blotting immunoassay. Int Arch Allergy Appl Immunol 1985; 78:300-4.
- 9 Kahn CR, Marsh DG. Monoclonal antibodies to the major *Lolium perenne* (rye grass) pollen allergen, *Lol p I* (Rye I). Mol. Immunol 1986; 23:1281-8.
- 10 Mourad W, Mécheri S, Peltre G, David B, Hébert J. Study of the epitope structure of purified *Dac g I* and *Lol p I*, the major allergens of *Dactylis glomerata* and *Lolium perenne* pollens, using monoclonal antibodies. J Immunol 1988; 141:3486-91.
- 11 Standring R, Spackman V, Porter SJ. Distribution of a major allergen of rye grass (*Lolium perenne*) between other grass species. Int Arch Allergy Appl Immunol 1987; 83:96-103.
- 12 Esch RE, Klapper DG. Cross-reactive and unique grass group I antigenic determinants defined by monoclonal antibodies. J Allergy Clin Immunol 1987; 79:489-95.
- 13 Johnson P, Marsh DG. The isolation and characterization of allergens from the pollen of rye grass (*Lolium perenne*). Eur Polymer J 1965; 1:63-77.

- 14 Marsh DG. Allergens and the genetics of allergy. In: Sela M, ed. *The Antigens*, vol. 3. London: Academic Press, 1975:271-359.
- 15 Howlett BJ, Clarke AE. Isolation and partial characterization of two antigenic glycoproteins from rye-grass pollen. *Biochem J* 197;695-706.
- 16 Cottam GP, Moran DM, Standing R. Physicochemical and immunochemical characterization of allergenic proteins from rye grass (*Lolium perenne*) pollen prepared by a rapid and efficient purification method. *Biochem J* 1986; 234:305-10.
- 17 Mécheri S, Peltre G, David B. Purification and characterization of a major allergen from *Dactylis glomerata* pollen: the Ag Dg 1. *Int Arch Allergy Appl Immunol* 1985; 78:283-9.
- 18 Lin Z, Ekramoddoullah AKM, Kisil FT, Herbert J, Mourad W. Isolation and characterization of *Poa p* I allergens of Kentucky bluegrass pollen with a murine monoclonal anti-*Lol p* I antibody. *Int Arch Allergy Appl Immunol* 1988; 87:294-300.
- 19 Mourad W, Pelletier G, Boulet A, Islam N, Valet J-P, Hébert J. Allergenicity and cross-reactivity of rye grass pollen extracts revealed by monoclonal antibodies. *J Immunol Methods* 1986; 89:53-9.
- 20 Matthiesen F, Schumacher M, Løwenstein H. Characterization of the major allergen of *Cynodon dactylon* (Bermuda grass) pollen. *J Allergy Clin Immunol* 1988; 81:266 (Abstract).
- 21 Ekramoddoullah AKM, Kisil FT, Schon AH. Immunochemical characterization of a high-molecular-weight basic allergen (HMBA) or ryegrass (*Lolium perenne*) pollen. *Mol Immunol* 1983; 20:465-73.
- 22 Haavik S, Paulsen BS, Wold JK. Glycoprotein allergens in pollen of timothy. II. Isolation and characterization of a basic glycoprotein allergen. *Int Arch Allergy Appl Immunol* 1985; 78:260-8.
- 23 Ansari AA, Shenbagamurthi P, Marsh DG. Complete amino acid sequences of *Lol p* II and *Lol p* III. *J Allergy Clin Immunol* 1989; 83:251 (Abstract).
- 24 Lowry OH, Rosebrough NJ, Fair AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; 193:265-75.
- 25 Ceska M, Eriksson R, Varga TM. Radioimmunosorbant assay of allergens. *J Allergy Clin Immunol* 1972; 49:1-9.
- 26 Lewis WH, Vinay P, Zenger VE. *Airborne and allergenic pollen of North America*. Boston: The Johns Hopkins University Press, 1983.
- 27 Løwenstein H, Wihl J-Å, Billesbølle KB, Bøwadt H. Rationale for specific immunotherapy of grass pollen allergy with extracts of rye pollen. *Allergy* 1984; 39:421-32.
- 28 Renck B, Einarsson R. Crossed radioimmunoelectrophoretic analysis of cultivated rye (*Secale cereale*) pollen allergens. *Int Arch Allergy Appl Immunol* 1984; 73:193-7.
- 29 Matthiesen F, Schumacher MJ, Løwenstein H. An immunoelectrophoretic analysis of the allergens of *Cynodon dactylon* (Bermuda grass) pollen. *J Allergy Clin Immunol* 1989; 83:1124-34.
- 30 Schumacher M, Griffith RD, O'Rourke MK. Recognition of pollen and other particulate aeroantigens by immunoblot microscopy. *J Allergy Clin Immunol* 1988; 82:608-16.
- 31 Matthiesen F, Klysner S, Løwenstein H. Monoclonal antibodies against group I and group V allergens in grass pollens. *Clin Exp Allergy* 20(Suppl. 1):47 (Abstract).
- 32 Ipsen H, Larsen JN. Detection of antigen-specific IgE antibodies in sera from allergic patients by SDS-PAGE immunoblotting and crossed radioimmunoelectrophoresis. In: Bjerrum OJ, Heegaard NHH, eds. *Handbook of immunoblotting of proteins*, Vol. 2. New York: CRC Press, 1988:159-66.
- 33 Matthiesen F, Løwenstein H. Characterization of the major allergens of *Phragmites communis* (reed) pollen. *J Allergy Clin Immunol* 1987; 79:215 (Abstract).
- 34 Marsh DG, Goodfriend L, King TP, Løwenstein H, Platts-Mills TAE. Allergen nomenclature. *J Allergy Clin Immunol* 1987; 80:639-45.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.